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Comparison of Several Fixation Methods for Cytomegalovirus Antigenemia Assay

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Two fixation methods based on formaldehyde or acetone for qualitative cytomegalovirus antigenemia assay were evaluated on 405 consecutive blood samples. Cytomegalovirus was detected in 40 samples by the antigenemia assay: 36 were detected by formaldehyde fixation; 22, by acetone; and 18, by both methods. Differences were statistically significant ($P = 0.0043$). In addition, four fixation methods (two based on formalin [with and without permeabilization] and two using acetone at different fixation times) for quantitative antigenemia assay in a different set of 32 samples from known viremic patients were evaluated. Formalin-based methods were superior to acetone-based methods, showing statistically significant differences in either the number of positive samples detected ($P < 0.02$; McNemar test) or the mean positive cell counts ($P < 0.003$; two-tailed Student's *t* test for paired samples). No differences between the two formalin-based methods were found. We recommend the formaldehyde fixation procedure without subsequent permeabilization because of its simplicity and sensitivity.

Cytomegalovirus (CMV) is a major pathogen in immunocompromised patients such as organ transplant recipients and those suffering from AIDS. The clinical picture of CMV disease, however, is indistinguishable from those of other opportunistic infections. Although this virus can be detected in several clinical specimens, thus allowing the diagnosis of a CMV active infection, detection of CMV in blood is more closely associated with the clinical manifestations of CMV disease (13). In addition, polymorphonuclear leukocytes appear to be the major carriers of CMV in blood (4). With the advent of antiviral therapy against CMV, there is a need for a rapid and sensitive method for the diagnosis of CMV infections and disease.

Among the different techniques available for CMV detection, the antigenemia assay is a step above other techniques because of its simplicity, sensitivity, and specificity (1, 5, 6, 15, 18, 20). In addition, higher numbers of leukocytes expressing the CMV antigens (quantitative antigenemia assay) correlated well with symptomatic infections or CMV disease (8, 10, 11, 16). Despite these advantages, it is evident from the literature that there is considerable variation in quantitative results among different reports, so the assay needs technical optimization and standardization (3, 7, 19, 22).

Several factors have been reported to influence the test performance, including the type of CMV antigen to be detected, the fixation method, and the immunostaining technique. Of the different antigens, the pp65 CMV low-matrix phosphoprotein seems to be the most suitable for these purposes (3, 19, 22). As for the fixation procedure, water-free acetone has been used in most studies as well as in a commercially available kit for antigenemia (CMV-vue; Incstar) (5, 11, 15, 16, 20, 21). However, two studies have reported better results with formalin-based fixation methods (3, 7). We carried out a collaborative study using two different prospective study designs to establish the test performance of formalin-based fixation methods in comparison with acetone-based methods.

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Study design and specimens. We carried out two different study protocols. The first protocol (part A), performed at Hospital Covadonga, was designed as a qualitative, prospective field trial on 405 blood samples from 113 patients at risk for CMV disease (61 renal transplant recipients, 41 AIDS patients, 4 bone marrow recipients, and 7 miscellaneous patients).

In the second part of the study (part B), carried out at Hospital Prínceps d'Espanya, we prospectively compared four different fixation techniques on 32 blood samples from 18 patients (6 liver transplant recipients, 5 renal transplantees, 6 AIDS patients, and 1 patient suffering from chronic renal insufficiency who was receiving therapy with corticoids) with a high probability of contracting CMV viremia (a positive antigenemia test and/or a positive shell vial result in one specimen collected within the two previous days). Half of these samples were obtained during ganciclovir therapy from six patients suffering from CMV disease (one each with hepatitis, pneumonitis, retinitis, and pancreatitis and two with symptomatic viremia). Two of the fixation procedures tested in part B used acetone at different fixation times, and the other two procedures were based on a 5% formalin solution used alone or in conjunction with permeabilization. The second study was designed to establish quantitative differences in CMV-positive cell counting between the different fixation methods, so a quantitative procedure was followed. In addition, the quality of the microscopic image was recorded in each preparation used in part B.

Leukocyte extraction and slide preparation. We used the dextran sedimentation method for leukocyte extraction, as previously described (14), with minor modifications. Briefly, 5 to 10 ml of heparinized whole blood was mixed with 1 ml of 6% dextran solution in saline (Macrodex; Pharmacia, Uppsala,

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Sweden), and the mixture was allowed to sediment at 37°C for 20 to 30 min. The leukocyte-rich fraction (overall, 70 to 80% of polymorphonuclear cells in our experience) of the supernatant was then collected and centrifuged at low speed, and contaminating erythrocytes were lysed by suspending the sediment in a chilled 0.8% ammonium chloride solution. After being washed in phosphate-buffered saline, pH 7.2 (PBS), the cell suspension was again centrifuged and the sediment was resuspended in PBS. For the quantitative part of the study, cells in the washed suspension were counted in a hematological counter and then adjusted to 10^6 cells per ml with PBS.

In part A of the study (qualitative assay), one 10-μl drop of the leukocyte suspension was placed in duplicate wells of a fluorescence slide, and the slide was allowed to dry at room temperature. Another well with cells was included in each sample to control the fluorescent conjugate. In part B, a total of 200 μl of the adjusted suspension (ca. 10^5 cells per spot) was spotted onto slides with a cytocentrifuge (Cytospin 3; Shandon Scientific, Runcorn, England) at 700 rpm for 7 min, and then slides were allowed to dry at room temperature. In both parts of the study, known positive specimens were included as controls when appropriate.

Fixation and staining methods. The following fixation methods were used during the study. (i) The acetone-based methods used water-free cold acetone (−20°C) for 20 min (method A20m; part A), 10 min (A10m; part B), and 90 s (A90s; part B). After fixation, slides were dried at room temperature. (ii) For formalin fixation (method F; part B), slides were immersed in a 5% *p*-formaldehyde–2% sucrose solution in PBS for 10 min, washed in PBS for 3 min, and allowed to dry. (iii) Formalin fixation plus permeabilization (method FP; parts A and B) was as follows: after immersion in the formalin solution described above, slides were washed in PBS containing 1% fetal calf serum and then permeabilized in PBS with 10% sucrose–0.5% Nonidet P-40 (Sigma Chemical, St. Louis, Mo.)–1% fetal calf serum for 5 min. Slides were then rinsed in PBS containing 1% fetal calf serum and allowed to dry.

After fixation, slides were stained by an indirect immunofluorescence assay with a commercially available murine monoclonal antibody directed against CMV pp65 (Monofluokit CMV; Diagnostics Pasteur, Marnes-la-Coquette, France) and a fluorescence-conjugated sheep anti-mouse immunoglobulin included in the same commercial kit. Slides were read under fluorescence microscopy. In part B of the study, quantitative results were expressed as the number of CMV-positive cells per 10^5 total leukocytes.

Other virological studies. Tube and shell vial cultures were performed on MRC-5 fibroblast monolayers in all blood samples, in accordance with standard procedures (9, 17). Vials were inoculated with 3×10^5 to 4×10^5 leukocytes, centrifuged at $700 \times g$ for 40 min, incubated at 37°C for 18 to 24 h, and then stained by an indirect immunofluorescence assay method with a monoclonal antibody directed against the 72-kDa immediate-early CMV antigen (E13; Argene Biosoft, Varilhes, France). Inoculated tubes were incubated at 37°C for at least 15 (part B) or 30 (part A) days and checked four times per week for the typical CMV cytopathic effect.

Statistical analysis. The McNemar test was used to compare the differences in the number of positive specimens for each fixation method. To compare the mean positive cell counts (part B of the study), the two-tailed Student's *t* test for paired samples was used after analysis of variance was carried out. A *P* value of less than 0.05 was considered statistically significant.

Results from part A of the study (field study) are shown in Table 1. CMV was detected in 46 of the 405 blood samples (11.4%): 40 were positive by the antigenemia test and 32 were positive by culture methods (tube and/or shell vial cultures). The positive specimens were obtained from 15 patients (13 renal transplant recipients and 2 AIDS patients). Of the 40 antigenemia-positive results, 36 were obtained after fixation with formaldehyde (FP method); acetone fixation (A20m method) was able to detect CMV in 22 blood samples. In 18 specimens, the antigenemia test was positive only after the FP fixation method had been applied. Conversely, four samples were only positive by the A20m method. These differences were statistically significant ($P = 0.0043$; McNemar test).

TABLE 1. CMV detection by the antigenemia assay and culture techniques in 405 blood samples from patients at risk (part A of study) and comparison of two fixation procedures

Determination	No.	%
Total CMV-positive blood samples	46	100.0
Positive by antigenemia assay	40	87.0
Formaldehyde fixation (FP method)	36	78.3
Acetone fixation (A20m method)	22	47.8
Positive only by FP method	18	39.1
Positive only by A20m method	4	8.7
Positive by culture technique	32	69.6
Tube culture	24	52.2
Shell vial culture	22	47.8

As for the specificity of the antigenemia assay in part A, 14 antigenemia-positive samples were not confirmed by either tube culture or the shell vial technique. All were from patients with culture-confirmed CMV viremia in other specimens drawn within a period of 1 month, and a significant part of these positive specimens were obtained during ganciclovir therapy or immediately afterwards. Conversely, there were six samples from six patients positive only by culture methods: three of them were from patients with positive antigenemia test results in subsequent blood samples.

A total of six patients from part A of the study developed clinically relevant CMV infection (CMV disease). Five of them presented with symptomatic viremia (fever and leukopenia), and the other presented with CMV pneumonitis (histopathologically confirmed at autopsy) and viremia. Three of these patients also presented with transaminitis, but CMV hepatitis was not confirmed due to the lack of biopsy specimens. Four of these six patients were treated with ganciclovir, with good clinical and virological response except for the patient with pneumonitis, who died. The two remaining patients recovered after decreasing azathioprine dosages. In all patients with CMV disease, the antigenemia test results were positive at the beginning of the symptoms. In four of these patients, viremia was also detected. In the remaining two, early ganciclovir therapy could have affected CMV recovery in cultures.

Table 2 shows the results from part B of the study. As expected, all blood samples were positive for CMV antigenemia by at least one of the fixation methods. Three of the samples were positive only by one method with quantitations of 1 (two samples) and 2 CMV-positive cells per 10^5 total leukocytes (one sample). Both formalin-based fixation procedures showed statistically significant differences in the number of positive specimens compared with each acetone-based method ($P < 0.02$ in all cases). No significant differences were shown between methods F and FP or between methods A90s and A10m. As for positive cell counts, the two methods using formaldehyde as a fixative were superior to those based on acetone in both absolute numbers and mean positive cell counts. Methods F, FP, A90s, and A10m gave the highest CMV-positive cell counts in 19, 20, 1, and 1 sample, respec-

TABLE 2. Quantitative CMV antigenemia results of different fixation procedures in 32 blood samples from known viremic patients (part B of study)

Fixation method	No. of positive specimens		CMV-positive cells/10^5 leukocytes	
	Total (%)	By this method only	Range	Mean
Formaldehyde, 5%	30 (93.8)	1	0–>250	61.3
Formaldehyde-permeabilization	30 (93.8)	1	0–>250	57.8
Acetone, 90 s	23 (71.9)	0	0–123	16.1
Acetone, 10 min	22 (68.8)	1	0–88	12.2

tively. Differences in mean positive cell counts were statistically significant when both F and FP methods were compared with either the A90s or the A10m method ($P < 0.003$). No differences in mean positive cell counts were observed between methods F and FP or methods A90s and A10m. Finally, all preparations fixed with the two formalin-based procedures showed a well-defined and more intense fluorescence pattern in cells expressing the pp65 CMV antigen. In contrast, a more diffuse pattern was observed in the vast majority of the preparations fixed with acetone.

Tube cultures confirmed a total of 16 cases (50.0%) from part B of the study, including 5 of the 16 specimens collected during ganciclovir therapy for confirmed CMV disease. Shell vial cultures confirmed a total of 19 of 32 samples (59.4%), 6 in specimens from treated patients. In total, 22 samples were positive for CMV by one of the culture methods. All except 1 of the 10 antigenemia-positive, culture-negative blood samples were from patients being treated with ganciclovir. The remaining sample was obtained from an untreated patient who had culture-confirmed viremia in one specimen drawn 25 days before the study, as well as other positive antigenemia tests within the study period.

Since its initial report in 1988 (21), the CMV antigenemia assay has appeared as a major advance in the diagnosis of CMV active infections and, more interestingly, CMV disease. Among the different advantages of this method, we should note its rapidity (4 to 5 h of process time), reliability (both sensitivity and specificity), and simplicity (no need for cell culture or other sophisticated technologies). In addition, pp65 antigenemia has proved to be an earlier marker of cytomegalovirus infection than culture techniques and serology (8, 10, 16). However, the ease with which the antigenemia assay quantifies the presence of the virus in blood, thus offering us a valuable tool for the diagnosis and prognosis of CMV infection and for the monitoring of antiviral therapy, may be its most important feature. For all of these reasons, some authors have proposed this assay as the method of choice for the diagnosis and follow-up of patients at risk for CMV disease.

The type of fixative used for antigenemia assay seems to be one of the most important factors in test performance (2). Several protocols have been applied, those based on acetone being the most frequently reported in the literature, including the original description of the assay (21). Results from our study clearly demonstrate the superiority of formaldehyde fixation methods over those using acetone at different fixation times. The number of positive specimens detected in parts A and B of the study, as well as the CMV-positive cell counts (part B), was higher with the formalin-based methods, and differences were statistically significant in all comparisons. Our results are in agreement with other reports showing the superiority of formaldehyde solutions as fixatives for CMV antigenemia assay, either qualitatively or quantitatively (3, 7). In the study by Landry and Ferguson, however, no significant differences were found between formaldehyde and acetone when the antigenemia assay was used as a qualitative test (12). As for the quality of the microscopic image, our study also confirms that better results are obtained with formaldehyde fixation methods, resulting in superior cell morphology and improved readability. This is a constant in reports analyzing this aspect (3, 7, 12).

No significant differences were noted when simple formalin fixation was compared with formaldehyde fixative followed by permeabilization with a detergent solution, the method proposed by Gerna et al. (7), offering the possibility of simplifying the fixation protocol. However, no differences were found between fixation with acetone at different times in part B of the study either, so the suspicion that prolonged fixation times negatively affect test performance (2, 7) was not confirmed.

Although our studies were not specifically designed for these purposes, the results showed the high sensitivity and specificity of the antigenemia assay, in accordance with other reports (1, 5, 8, 15, 20). In both parts of the study, sensitivity was higher for the antigenemia assay than for culture techniques. Most antigenemia-positive, culture-negative results occurred in two instances: (i) with samples obtained during or immediately after antiviral therapy; and (ii) with blood specimens with low levels of viral load from known viremic patients, either at the beginning of the process or as a residual result. This confirms the high specificity of the assay and, partially, its usefulness in monitoring treated patients.

In summary, our results showed the superiority of fixation methods based on formaldehyde over those using acetone as a fixative. We recommend the formaldehyde fixation procedure without subsequent permeabilization because of its simplicity and sensitivity. We also confirm the excellent performance of the antigenemia assay.

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REFERENCES

1. **Aguado, S., E. Gómez, A. Rodríguez, A. Martínez A, M. Oña, and J. Alvarez-Grande.** 1989. A prospective study on a rapid method for diagnosis of cytomegalovirus infections in immunosuppressed patients. Nephron **53:**322–324.
2. **Bein, G., A. Bitsch, J. Hoyer, and H. Kirchner.** 1991. The detection of human cytomegalovirus immediate early antigen in peripheral blood leucocytes. J. Immunol. Methods **137:**175–180.
3. **Boeckh, M., P. M. Woogerd, T. Stevens-Ayers, C. G. Ray, and R. A. Bowden.** 1994. Factors influencing detection of quantitative cytomegalovirus antigenemia. J. Clin. Microbiol. **32:**832–833.
4. **Dankner, W. N., J. A. McCutchan, D. D. Richman, K. Hirata, and S. A. Spector.** 1990. Localization of human cytomegalovirus in peripheral blood leukocytes by in situ hybridization. J. Infect. Dis. **161:**31–36.
5. **Erice, A., M. A. Holm, P. C. Gill, S. Henry, C. L. Dirksen, D. L. Dunn, R. P. Hillam, and H. H. Balfour, Jr.** 1992. Cytomegalovirus (CMV) antigenemia assay is more sensitive than shell vial cultures for rapid detection of CMV in polymorphonuclear blood leukocytes. J. Clin. Microbiol. **30:**2822–2825.
6. **Freymuth, F., E. Gennetay, J. Petitjean, G. Eugene, B. Herault de Ligny, J. P. Ryckelynck, C. Legoff, P. Hazera, and C. Bazin.** 1994. Comparison of nested PCR for detection of DNA in plasma with pp65 leukocytic antigenemia procedure for diagnosis of human cytomegalovirus infection. J. Clin. Microbiol. **32:**1614–1618.
7. **Gerna, G., M. G. Revello, E. Percivalle, and F. Morini.** 1992. Comparison of different immunostaining techniques and monoclonal antibodies to the lower matrix phosphoprotein (pp65) for optimal quantitation of human cytomegalovirus antigenemia. J. Clin. Microbiol. **30:**1232–1237.
8. **Gerna, G., D. Zipeto, M. Parea, M. G. Revello, E. Silini, E. Percivalle, M. Zavattoni, P. Grossi, and G. Milanesi.** 1991. Monitoring of human cytomeg-

alovirus infections and ganciclovir treatment in heart transplant recipients by determination of viremia, antigenemia and DNAemia. J. Infect. Dis. **164:** 488–498.

9. **Gleaves, C. A., T. F. Smith, E. A. Shuster, and G. R. Pearson.** 1985. Comparison of standard tube and shell vial cell culture techniques for the detection of cytomegalovirus in clinical specimens. J. Clin. Microbiol. **21:**217–221.
10. **Gómez, E., S. Aguado, S. Melón, M. Martínez, M. De Oña, R. Cimadevilla, M. Gorostidi, E. González, and J. Alvarez-Grande.** 1992. A prospective study of the usefulness of rapid diagnostic tests in monitoring cytomegalovirus infection in renal transplant. Transplant. Proc. **24:**2631–2633.
11. **Koskinen, P. K., M. S. Nieminen, S. P. Mattila, P. J. Hayry, and I. T. Lautenschlager.** 1993. The correlation between symptomatic CMV infection and CMV antigenemia in heart allograft recipients. Transplantation **55:**547–551.
12. **Landry, M. L., and D. Ferguson.** 1993. Comparison of quantitative cytomegalovirus antigenemia assay with culture methods and correlation with clinical disease. J. Clin. Microbiol. **31:**2851–2856.
13. **Meyers, J. D., P. Ljungman, and L. D. Fisher.** 1990. Cytomegalovirus excretion as predictor of cytomegalovirus diseases after marrow transplantation: importance of cytomegalovirus viremia. J. Infect. Dis. **162:**373–380.
14. **Niubò, J., J. L. Pérez, A. Carvajal, C. Ardanuy, and R. Martín.** 1994. Effect of delayed processing of blood samples on the performance of cytomegalovirus antigenemia assay. J. Clin. Microbiol. **32:**1119–1120.
15. **Pérez, J. L., D. Mariscal, F. Tubau, J. Niubò, and R. Martín.** 1994. Evaluation of the CMV-vue™ antigenemia assay for rapid detection of cytomegalovirus in peripheral blood leucocytes. Diagn. Microbiol. Infect. Dis. **19:**15–18.
16. **Revello, M. G., E. Percivalle, M. Zavattoni, M. Parea, G. Grossi, and G. Gerna.** 1989. Detection of human cytomegalovirus immediate early antigen in leukocytes as a marker of viremia in immunocompromised patients. J. Med. Virol. **29:**88–93.
17. **Stagno, S., W. J. Britt, and R. F. Pass.** 1989. Cytomegalovirus, p. 321–378. *In* N. J. Schmidt and R. W. Emmons (ed.), Diagnostic procedures for viral, rickettsial and chlamydial infections. American Public Health Association, Washington, D.C.
18. **Storch, G. A., R. S. Buller, T. C. Bailey, N. A. Ettinger, T. Langlois, M. Gaudreault-Keener, and P. Welby.** 1994. Comparison of PCR and pp65 antigenemia assay with quantitative shell vial culture for detection of cytomegalovirus in blood leukocytes from solid-organ transplant recipients. J. Clin. Microbiol. **32:**997–1003.
19. **The, T. H., M. Van der Ploeg, A. P. Van den Berg, A. M. Vlieger, M. Van der Giessen, and W. J. Van Son.** 1992. Direct detection of cytomegalovirus in peripheral blood leukocytes—a review of the antigenemia assay and polymerase chain reaction. Transplantation **54:**193–198.
20. **Van den Berg, A., W. Van der Bij, W. J. Van Son, J. Anema, M. Van der Giessen, J. Schirm, A. M. Tegzess, and T. H. The.** 1989. Cytomegalovirus antigenemia as a useful marker of symptomatic cytomegalovirus infection after renal transplantation: a report of 130 consecutive patients. Transplantation **48:**991–995.
21. **Van der Bij, W., R. B. Van Dijk, W. J. Van Son, R. Torensma, K. B. Prenger, J. Prop, A. M. Tegzess, and T. H. The.** 1988. Antigen test for early diagnosis of active cytomegalovirus infection in heart transplant recipients. J. Heart Transplant. **7:**106–110.
22. **Van der Giessen, M., T. H. The, and W. J. Van Son.** 1991. Cytomegalovirus antigenemia assay. J. Clin. Microbiol. **29:**2909–2910. (Letter.)